Seven-Day Administration of Recombinant Human Granulocyte Colony-Stimulating Factor to Newborn Rats: Modulation of Neonatal Neutrophilia, Myelopoiesis, and Group B Streptococcus Sepsis

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Single-pulse administration of rhG-colony-stimulating factor (CSF) to neonatal rats was previously demonstrated to induce peripheral neutrophilia and modulate bone marrow (BM) neutrophil storage and proliferative pools (NSP + NPP). In this study, we investigated the prolonged effects of 7 days of rhG-CSF therapy (5 µg/kg/day). Sprague-Dawley newborn rats (±24 hours) were injected intraperitoneally (IP) (daily for 7 days) with rhG-CSF or phosphate-buffered saline/human serum albumin (PBS/HSA). RhG-CSF induced a significant early and late peripheral neutrophilia: 6,905 ± 1,625 (day 1) and 9,223 ± 515 µL (day 7) vs 1.275 ± 90/µL (P ≤ .0001). In addition, 7 days of rhG-CSF resulted in a significant increase in the BM NSP: 3,247 ± 190/µL vs 1,677 ± 339/µL (P ≤ .001). There was, however, no depletion or significant change in the BM NPP. Seven days of rhG-CSF also induced a mild increase in BM CFU-GM colony formation (P ≤ .01). There was, however, no significant change in liver/spleen CFU-GM colonies or in the CFU-GM proliferative rate in either the BM or liver/spleen cultures. Finally, 7 days of prophylactic rhG-CSF therapy resulted in a synergistic response with antibiotic therapy and significantly modulated the mortality rate during experimental group B streptococcal sepsis (GBS) (100% vs 50%) (GvsC) (P ≤ .001). Pulse rhG-CSF administered at 6 hours or 18 hours after GBS inoculation, however, failed to act synergistically with antibiotics to improve survival or prevent peripheral neutropenia. This study suggests that 7 days of prophylactic rhG-CSF therapy induces peripheral neutrophilia, myeloid maturation, increases neutrophil BM reserves and also may provide immunologic enhancement of neonatal host defense during experimental GBS in term newborn rats.

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**MATERIALS AND METHODS**

**G-CSF.** Recombinant human G-CSF was obtained from Amgen Biologicals, Thousand Oaks, CA, and was prepared from an E. coli host recombinant DNA procedure to greater than 95% purity before formulation in 0.025% human serum albumin (HSA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm the purity of the growth factor by the appearance of a single protein band. Biologic activity of 2 x 10^6 U/mg was determined by granulocyte colony formation (CFU-G) on human nonadherent BM cells in semisolid media. The Limulus amebocyte lysate assay was used to demonstrate the absence of measurable endotoxin. Purified G-CSF was used at concentrations of 5.0 µg/kg (diluted with phosphate-buffered saline (PBS), pH 7.4, and HSA, (Alpha Therapeutic, Los Angeles, CA).

**Organism.** GBS, type III, Norris, was provided by Dr Gerald Norris, Children’s Hospital of Orange County, Orange, CA 92668.
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Fisher (Bethesda, MD). The organism was isolated from an infected neonate and serotyped by the precipitin method using rabbit antisera. The organism was grown in Todd-Hewitt broth (THB) BBL Microbiology Systems, Cockeysville, Maryland, to logarithmic phase and then aliquoted and stored at −70°C until use. Aliquots were thawed and allowed to grow to maximum phase growth in fresh THB. Organisms were then sedimented by centrifugation and washed three times in sterile PBS. Concentration of bacteria was standardized by its optical density at 620 nm, and a suspension of 3 × 10⁸ organisms per g body weight per 0.100 mL was prepared for injection.

Animal inoculation. Litters of albino Sprague-Dawley neonatal rats (Bantin-Kingman Laboratories, Fremont, CA) 24 hours (6 to 8 g) were used during this study. Mothers of the litters were received 1 week before delivery and were housed at the vivarium at the University of California Irvine Medical Center. They were maintained at constant room temperature, with water and rodent feed (Purina Chow) ad libitum. Approval for this study was granted by the University Animal Use Committee. Before the neonatal rats were inoculated, the site of injection was washed with Betadine solution (povidone-iodine, 10%, Purdue Frederick, Norwalk, CT).

Daily (7 days) IP injections of G-CSF were accomplished with a sterile tuberculin syringe fitted with a 27.5-gauge needle. Antibiotics were administered intramuscularly (IM) in the hind leg. GBS was inoculated subcutaneously (SC) in the tail region of the animal with a sterile tuberculin syringe.

Quantification of circulating, proliferative, and storage pools. Blood samples were obtained daily by nicking the jugular vein with a sterile scalpel, and 10 μL free-flowing blood was collected. Samples were electronically counted (Sereno-Baker Diagnostics, Allentown, PA), blood smears were prepared and stained with Wright’s stain, and a 100 to 200 cell differential was performed. Absolute neutrophil counts were determined by multiplication of the nucleated cell count by the percentage of neutrophils in the differentials. BM NSP and NPP were determined on day 8 by the method of Christensen et al.⁵ Neonatal femurs were aseptically removed, and the contents were flushed into a known quantity of Hanks’ balanced salt solution (HBSS, GIBCO, Grand Island, NY). Livers and spleens were also removed on day 8 in a similar manner and finely minced in a known quantity of HBSS. Electronic cell counts were performed on the BM, and 500-cell differential count was obtained on Wright-stained cytospin preparations.

CFU-GM proliferative rate. Proliferative rates of CFU-GM were evaluated by the thymidine suicide method of Christensen et al.⁶ BM and combined liver and spleen cells from neonatal rats were placed into each of two 50-mL centrifuge tubes. To the first aliquot was added 0.34 μg nonradioactive thymidine in a 0.1-mL vol. The other aliquots received 0.34 μg methyl-³H-thymidine containing 0.1 mCi (specific activity, 75 Ci/mmol, ICN Radiochemicals, Irvine, CA). The tubes were incubated for 20 minutes at 37°C while being agitated every 5 minutes. Thymidine uptake was halted by adding 30 mL of ice-cold α-minimum essential medium (α-MEM) with 5% fetal calf serum (FCS) and 100 μg/mL nonradioactive thymidine. The cell suspensions were then centrifuged and washed twice with the α-MEM/FCS/thymidine media. Cells were then added in the same 1.1% methylcellulose/α-MEM described above. Colonies were allowed to develop for 14 days in a 5% CO₂ incubator at 37°C, and thymidine suicide rate was determined by subtracting the average number of colonies formed per plate by cells exposed to ³H-thymidine from the average number of colonies per plate formed by cells exposed to nonradioactive thymidine divided by the average colonies per plate from cells exposed to nonradioactive thymidine.

Septis studies. The 90% lethal dose (LD-90) at 48 to 72 hours after GBS inoculation was determined by injecting neonatal rats as described above with concentrations of 3 × 10⁸, 3 × 10⁹, 3 × 10¹⁰, and 3 × 10¹¹ bacteria per gram of weight. To determine the effect of G-CSF, litters were injected with IP G-CSF (5.0 μg/kg), or PBS/0.025% HSA. Litters each received one of several forms of therapy and were inoculated with GBS (1 × 10⁸ bacterial per gram on day 8): (a) 7-day rhG-CSF, administered IP at 5.0 μg/kg, (b) 7-day 0.025% PBS/HS aminoglycoside IP; (c) 7-day rhG-CSF and antibiotics, ampicillin (Bristol Laboratories, Evansville, IN), 150 mg/kg per day, and gentamicin (Elkins-Sinn, Cherry Hill, NJ), a total of 6.5 mg/kg per day IM divided in two doses starting 24 hours after infection with GBS; and (d) 7 days PBS/0.025% HSA and similar antibiotic therapy. In addition, 1-day-old newborn rats were inoculated with GBS and treated with combinations of rhG-CSF given at either 18 hours after GBS inoculation or at 6 hours (every 12 hours) after GBS and treated with or without additional antibiotics 24 hours after GBS. Mortality and morbidity were monitored throughout the sepsis studies.

CFU-GM colony quantification. BM and liver/spleen cells were collected as described above. Cells were then suspended in α-MEM (GIBCO) with 1.1% methylcellulose (Terry Fox Laboratories, Logan, UT), 10% bovine serum albumin (BSA) (Sigma) 14.3 × 10⁻³ mol/L mercaptoethanol, 0.01% vol/vol murine spleen cell conditioned medium pokeweed mitogen (PWM) SCCM, (Terry Fox Laboratories), and erythropoietin 1 U/mL (Amgen). Penicillin 100,000 U/L and streptomycin 100 mg/L (P/S) were also added. Cell suspensions were plated in triplicate in 10 × 35-mm tissue culture dishes (Nunc, Denmark) and incubated at 5% CO₂, 37°C, in a high-humidity atmosphere. Cultures were evaluated at 14 days. Aggregates of more than 50 cells were considered “colonies.” Colonies were plucked at random, placed on a slide and stained, and specific lineage was confirmed. Results are reported total cells per marrow and total cells per liver/spleen suspension.

Statistical analysis. All results are expressed as mean values plus or minus SEM of 8 to 15 animals, or three to five replicates of blood, BM, or liver/spleen samples. The probability of significant differences when two treated groups were compared was determined with the unpaired Student's t test; and the probability of significant differences when multiple treatments were examined was determined by analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple-range tests to define the unique subsets within the study. Statistical analyses were performed with the Biostat 1 statistical program (Sigma Soft, Placentia, CA) for the IBM personal computer. P values <.05 were considered significant.

RESULTS

Complete blood counts and differentials were obtained on days 1, 3, 5, and 7 after IP rhG-CSF administration. Within 1 day of rhG-CSF administration, there was a very significant early peripheral neutrophilia (ANC): (6,905 ± 1,625/μL) versus (1,278 ± 90/μL) (G v C) (P < .004) (Fig 1). A continuous and prolonged neutrophilia occurred throughout the 7 days of G-CSF administration. On day 7, the ANC was still significantly higher than controls and significantly higher than after the first day of G-CSF therapy: 9,223 ± 515 v 5,151 ± 110/μL (P < .002) (day 7, GvC (Fig 1) and 9,223 ± 515 v 6,905 ± 1,905/μL) G-CSF: (day 7 v day 1) (P < .01). Neither were there significant differences in the peripheral lymphocyte, monocyte, or eosinophil counts during the 7 days of G-CSF therapy (data not shown).

The BM NSP was determined on day 8 after 7 days of IP G-CSF or IP PBS/0.025% HSA. The BM NSP was determined by calculating the total number of PMN + bands + metamyelocytes/μL. After seven daily injections of IP rhG-
were electronically counted. and a 100 to 200 cell differential was performed on Wright’s stained blood smears. Values are the mean ± SEM of 20 animals. ANC: G-CSF v PBS/HSA (P < .001).

CSF, the NSP was significantly increased in the G-CSF-treated group of animals as compared with the control group (P < .001). (Fig 2). We also calculated the BM NPP (blasts + promyelocytes + myelocytes) after 7 days of IP G-CSF and PBS/0.025% HSA. The BM NPP, however, was not significantly different in the G-CSF-treated group as compared with the control group of animals. (1,930 ± 32 v 1,570 ± 179/μL) (G v C), P = NS.

BM and liver/spleen CFU-GM were also determined on day 8 after 7 days of IP rhG-CSF. Total BM CFU-GM/femur was significantly higher in the G-CSF–treated group as compared with controls: 2,202 ± 248 versus 3,192 ± 428 (C v G) (P <.01). There was no difference in liver/spleen CFU-GM, however, between G-CSF–treated and non-treated groups: 3,702 ± 1,132 versus 4,568 ± 1,674 (G v C) (P = NS). With regard to progenitor proliferative rates, thymidine suicide studies were performed on myeloid progenitor cells from both the BM and liver/spleens from the day 8 BMs after 7 days of therapy. The BM proliferative rates trended to be higher in the G-CSF group but were not statistically significant (59.9% ± 9.8% v 53% ± 1.6%) (G v C). In addition, a similar increased trend was noted in the liver/spleen myeloid progenitor colonies: 61% ± 11.4% versus 52% ± 12% (G v C).

Morbidity and mortality were evaluated after 7 days of IP rhG-CSF or PBS/0.025% HSA and inoculation with GBS on day 8. Animals were weighed 72 hours after GBS inoculation. Neonatal rats pretreated with 7 days of IP rhG-CSF + antibiotics after GBS appeared the healthiest and weighed the most as compared with either the group treated with only rhG-CSF or PBS/0.025%/HSA + antibiotics. Even the animals that received rhG-CSF without antibiotics weighed more than the animals that received PBS/0.025% HSA and later antibiotic therapy after GBS: PBS/HSA + antibiotics 15 ± 0.28 g, rhG-CSF 17.4 ± 0.22 g, and rhG-CSF + antibiotics 19.1 ± 0.65 g (P <.05).

The mortality rate was also influenced by the 7-day pretreatment with rhG-CSF as compared with PBS/0.025% HSA–treated animals. Forty-eight hours after GBS inoculation, almost 100% of the control animals without subsequent antibiotic treatment were dead. This compared with a 60% death rate in the rhG-CSF–treated group and a 0% and 10% rate in the antibiotic-treated groups with or without rhG-CSF therapy, respectively. At 72 hours after inoculation of GBS, however, there was 0% survival in both the PBS/0.025% HSA group and the rhG-CSF group and only 50% survival in the PBS/0.025% HSA + antibiotics group. The group of animals pretreated with 7 days of rhG-CSF and then inoculated with GBS and subsequently given antibiotics, still had 100% survival, however (rhG-CSF + Ab v PBS/0.025% HSA + Ab) (100% v 50%) (P <.001) (Fig 3).

To delineate better the mechanism of synergism of rhG-CSF with antibiotics, newborn animals (1 day) were inoculated with GBS and randomized to rhG-CSF at either 6 hours or 18 hours ± antibiotics 24 hours after GBS. Addition of rhG-CSF at 18 hours after GBS did not induce an increase in survival over antibiotics alone (Fig 4). In addition, neither did administration of rhG-CSF at 6 hours and then every 12 hours thereafter modulate the survival rate v antibiotics alone (Fig 5). RhG-CSF 18 hours after GBS failed to prevent neutropenia after overwhelming GBS infection; ANC (μL) 6 hours after rhG-CSF treatment: no GBS 1,989 ± 484; rhG-CSF 160 ± 151; rhG-CSF + antibiotics 159 ± 60; antibiotics alone 155 ± 36; PBS/HSA 172 ± 25; P = NS. In addition, animals who survived after rhG-CSF treatment (6 hours after GBS every 12 hours) did not develop neutropenia, and their ANC was not statistically different from that of animals that received antibiotic therapy alone: ANC (μL) 2,108 versus 1923, (G v Ab).
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**Figure 3.** Litters of neonatal rats (≤24 hours) received rhG-CSF (or 0.025% HSA/PBS) IP for 7 days. Litters were inoculated with 1 x 10^9 GBS/g on day 8; 24 hours later, groups received antibiotics (ampicillin 150 mg/kg per day and gentamicin 6.5 mg/kg per day) every 12 hours IM or no additional treatment. Graph represents percentage of survival 96 hours after induced infection. RhG-CSF + antibiotics v PBS/HSA + antibiotics (P < .001).

**DISCUSSION**

At term, neonatal rat BM myeloid progenitor colonies (CFU-GM) are normally reduced to about 25% of adult rat levels and appear to be proliferating at 80% of capacity as compared with only a 25% proliferative rate in adult rats. In addition, neonatal rat BM NSP are substantially decreased to about 25% of normal adult rat levels. This immaturity in neonatal rat myeloid host defense results in an increased tendency to develop significant peripheral neutropenia during experimental overwhelming bacterial infection. Moreover, despite the presence of normal numbers of circulating mature effector granulocytes at the time of sepsis, the neonate may be still susceptible to overwhelming bacterial sepsis secondary to qualitative deficiencies within circulating phagocytes.

G-CSF has been reported to induce peripheral neutrophilia in mice, to shorten the period of neutropenia in cynomolus monkeys after cyclophosphamid and busulphan therapy, and to reduce morbidity successfully after BM transplantation. Recently, rhG-CSF was successfully used to shorten the period of neutropenia after multiagent chemotherapy in humans, and reverse cyclic neutropenia in both children and adults. The acute elevation of the peripheral neutrophil count within 48 hours after G-CSF therapy has been attributed to the release of BM NSP reserves, whereas the prolonged (7 to 10 day) increase in peripheral neutrophil counts after daily G-CSF therapy has been suggested to be secondary to the G-CSF stimulatory effects on BM myeloid progenitor cells.

In the present study, we demonstrated that rhG-CSF has similar significant effects on neonatal myelopoiesis. RhG-CSF, when administered for 7 consecutive days to term newborn rats, induced a profound neutrophilia within the first 24 hours and a sustained and increased response for the next 7 days. At the end of 7 days of rhG-CSF therapy, we also noted a significant increase (100%) in the BM NSP. This dramatic increase in the NSP 1 week after G-CSF therapy did not lead to a depletion of early BM myeloid progenitor cells (NPP), however. These findings are consistent with those of Ulich et al, who also reported a 100% increase in the NSP and no change in the NPP in adult rats treated with rhG-CSF for 7 consecutive days.

Erdman et al previously observed that the NSP of 1-week-old neonatal rats represented only 50% of normal

**Figure 4.** Litters of neonatal rats (≤24 hours) received rhG-CSF or 0.025% HSA/PBS IP 18 hours after infection with GBS. Litters were inoculated with 1 x 10^9 GBS/g at hour 0; 18 hours later, groups received rhG-CSF or HSA/PBS. Six hours later, groups received antibiotics (ampicillin 150 mg/kg per day and gentamicin 6.5 mg/kg per day) every 12 hours IM or no additional treatment. Graph represents the survival over a 96-hour period.
levels in adult animals. This study demonstrates that 7 days of rhG-CSF therapy to term neonatal rats resulted in increasing the NSP by 100% at age 1 week. This doubling of the NSP at age 1 week begins to approach the normal adult rat BM NSP reported by Erdman et al. The present study also showed that neonatal rat bone marrow myeloid progenitor colonies (CFU-GM) increased after 7 days of IP rhG-CSF without, however, any increase in liver/spleen CFU-GM. Christensen et al. previously demonstrated that 1-week-old neonatal rats possess only about 20% to 25% of normal adult rat CFU-GM colony activity. The lack of response in liver/spleen CFU-GM in this study is consistent with the hypothesis that with increasing age a progressive reduction occurs in CFU-GM colony activity in both liver and spleen pools in developing neonatal rats.

Broxmeyer et al. failed to document an increase in BM and spleen CFU-GM after pulse administration of rhG-CSF (1 to 2 x 10^6 U) to adult mice, but in higher doses of 10^6 U, both BM and spleen CFU-GM colony activity increased. Duhrsen et al. also noted that 5-day administration of rhG-CSF to adult cancer patients also failed to cause a significant increase in BM CFU-GM activity. In the present study, we also found no increase in the CFU-GM proliferative rate in either BM or liver/spleens after 7 days of IP rhG-CSF. Because the CFU-GM proliferative activity is already much higher in newborn rats as compared with adult animals, it is not surprising to fail to document a significant increase in this age group. These findings are somewhat different from those of Broxmeyer et al., who demonstrated an increase in CFU-GM S-phase activity in mouse BM and spleens after high-dose rhG-CSF and pretreatment with lactoferrin.

We also showed that 7 days of rhG-CSF in newborn rats modulates morbidity and mortality associated with overwhelming group B streptococcal sepsis. Seventy-two hours after GBS inoculation, there was still 100% survival in the rhG-CSF plus antibiotic-treated group as compared with survival of only 50% in the PBS/HSA plus antibiotic group. In addition, in the present study and as expected, 7 days of rhG-CSF therapy without subsequent antibiotic therapy did not enhance survival during GBS. Most important, however, rhG-CSF delayed onset of death as compared with PBS/HSA-treated control animals, suggesting that prophylactic rhG-CSF may enhance neonatal host defense by allowing the host more time to mount an immunologic response. We also noted a significant increase in the weight of animals treated with rhG-CSF + antibiotics as compared with their control groups. rhG-CSF administered 18 hours after GBS or 6 hours after GBS (every 12 hours) failed to prevent neutropenia or improve survival with supplemental antibiotic therapy, however. These combined studies suggest that 7 days of rhG-CSF therapy induces a significant peripheral neutrophilia and accumulation of BM NSP reserves. This increase in the BM NSP appears to prevent the NSP depletion that has been previously demonstrated with overwhelming GBS infection. Single or multiple pulses of rhG-CSF given after GBS inoculation, however, fail to sustain the peripheral neutrophil count, lead to significant neutropenia, and result in a high mortality rate.

Matsumoto et al. and Tanaka et al. also demonstrated the beneficial effects of rhG-CSF in adult cyclophosphamide-treated mice inoculated with *Pseudomonas* and of rmGM-CSF in cyclophosphamide-treated adult mice also inoculated with *Pseudomonas*. Recently, Silver et al. also reported significant synergistic activity of rhG-CSF plus gentamicin therapy in mice with *Pseudomonas* burn-induced sepsis.

The improvement in the mortality rate after 7 days of IP rhG-CSF presumably occurs secondary to stimulation of myeloid maturation; however, an enhancement of mature granulocyte effector function cannot be ruled out. G-CSF...
has previously been demonstrated to activate and enhance adult neutrophil physiologic function.\textsuperscript{14,15} We also recently demonstrated that rhGM-CSF stimulates in vitro neonatal neutrophil activity, including oxidative metabolism, bacterial killing, C3bi receptor expression, and adherence.\textsuperscript{31,32} Because in vitro neonatal rat neutrophil functional assays were not performed in this study, we cannot determine whether rhG-CSF may have modulated this area of host defense.

In summary, we demonstrated that prolonged (7 day) administration of IP rhG-CSF to term newborn rats results in significant alterations in BM myelopoiesis. Prolonged rhG-CSF therapy induces a maturation of early myeloid progenitor cells and an increase in mature NSP cells without resulting in a depletion of BM NPP. In addition, 7 days of rhG-CSF induces an early and sustained peripheral neutrophilia and a mild increase in BM CFU-GM activity. The resulting increase in BM NSP and peripheral neutrophilia after 7 days of IP rhG-CSF appears to modulate newborn rats’ response to experimental group B streptococcal sepsis.

Because premature newborns are even more immunologically immature than their term counterparts, further studies should be directed at investigating the role of these hematopoietic growth factors in modulating host defense during overwhelming bacterial sepsis in premature animals.

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REFERENCES

Seven-day administration of recombinant human granulocyte colony-stimulating factor to newborn rats: modulation of neonatal neutrophilia, myelopoiesis, and group B Streptococcus sepsis

MS Cairo, JM Plunkett, D Mauss and C Van de ven